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The tumor cell-endothelial-cell (EC) interaction, specifically that between E-selectin on EC and carbohydrate ligands on leukocytes and tumor cells, has recently emerged as a promising target for cancer therapy. The carbohydrate structures sialyl-Lewis X (SA-LeX), sialyl-Lewis a (SA-Le^a) and Lewis Y (LeY) are the key factor underlying the metastatic potential of carcinoma cells. The proposed studies explored the possibility of generating peptide mimetics to interrupt adhesion of tumor cells to vascular endothelium and thereby significantly reduce metastatic spread in analogy with reduction of neutrophil recruitment in chronic inflammation. We have identified a panel of 12-mer peptides from a random peptide library using monoclonal antibodies (MAbs) specific for human tumor-associated carbohydrate antigens, i.e., SA-Le^a, SA-LeX and LeY, and the peptides were proven to retain a carbohydrate-like conformation. Most importantly they can block selectin function in vivo. In addition to the originally proposed studies, we have identified a panel of peptides mimicking E-selectin ligand(s) using E-selectin-IgG immunoglobulin fusion protein and applied peptide array based on the selected peptides to characterize amino acids important for MAb/E-selectin interaction as well as to identify peptides with increased binding ability. We established syngeneic murine models and are in a process of applying the identified peptides as anti-adhesion therapy against development of metastasis. These studies provide significant information pertinent to new strategies for therapeutic intervention for human cancer and insight into the role of adhesion and carbohydrate determinants in the metastatic process.

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INTRODUCTION

Carbohydrate structures expressed on the surface of leukocytes and tumor cells mediate specific recognition by cell-surface selectins expressed on cytokine activated endothelial cells and platelets, underlying inflammation and metastasis, respectively. The intercellular interaction between EC and leukocytes and tumor cells represents a functional prototype of protein-carbohydrate interactions. Carbohydrate ligands such as SA-LeX and SA-Le^a are found on the surface of leukocytes and tumor cells, and their receptor, E-selectin, is expressed on activated endothelial cells. An objective of the proposed studies was to explore the possibility that interruption of protein-carbohydrate based adhesion of tumor cells to vascular endothelium reduces significantly metastatic spread in analogy with reduction of neutrophil recruitment in inflammatory conditions.

Specifically we proposed to use combinatorial libraries to identify structural equivalents or peptide mimotopes of carbohydrate ligands for E-selectin such as SA-Le^a and SA-LeX and E-selectin independent adhesion ligand LeY using specific MAbs and to investigate the effect of the identified peptides for their ability to interfere with tumor cell attachment in vitro using various adhesion assays and metastatic processes in vivo.

Aberrant glycosylation in tumors. Aberrant glycosylation in tumors relative to their normal counterparts represents a phenotypic feature associated with different human malignancies (1). This phenomenon has been demonstrated repeatedly at frequencies higher than those of oncogenes and suppressor genes in various tumors. Aberrant glycosylation is crucial in tumor progression, since cells acquire competence for metastasis and faster clonal growth via newly synthesized carbohydrate structures. Several types of altered glycosylation have been described in human carcinomas and among others enhanced expression of Lewis antigens such as sialyl-Le^a (SA-Le^a) [NeuAcα2,3Galβ1,3(Fucα1,4) GlcNAcβ1,3Galβ1,4Glcβ1-R] and sialyl-LeX (SA-LeX) [NeuAcα2,3Galβ1,4(Fucα1,3) GlcNAc β1,3 Galβ1,4Glcβ1-R] is observed more frequently than their respective nonsialylated forms (2-4). These structures were previously determined by us and others as being highly tumor-associated antigens in human adenocarcinomas. SA-LeX and SA-Le^a are recognized by members of the selectin endothelial adhesion molecules family and are postulated to be involved in metastatic spread.

Selectin-mediated adhesion of tumor cells. Selectins as a family of adhesion molecules are known to mediate critical cell-cell interactions in processes such as leukocyte trafficking, thrombosis, acute and chronic inflammation and ischemia reperfusion injury (5-7). Selectins support the adhesion of leukocytes to the vessel wall through the recognition of specific carbohydrate structures such as SA-LeX.

SA-LeX and its isomer SA-Le^a are tumor-specific oligosaccharides recognized by selectins and the metastatic potential of a variety of malignant cells is related to the functional expression of these ligands. Previous studies demonstrated that carcinoma cells bind to activated EC by E-selectin-dependent mechanism (8-11). Once tumor cells adhere to EC, they penetrate through the EC layer, moving into subendothelial tissues where metastasis is established. The expression of ligands for E-selectin by both neutrophils and carcinoma cells, raises the possibility that metastases are equivalent to the inflammatory process in which tumor cells, particularly carcinoma cells, use the same molecular mechanism(s) for cancer cell-EC interaction as lymphocytes, through the action of the endothelial selectins with the tumor-associated carbohydrate ligands, e.g. SA-LeX and SA-Le^a. Although adhesion pathways utilized by different tumors show considerable diversity, recent reports clearly suggest that at least one member of the selectin family of molecules and SA-Le^a and/or SA-LeX carbohydrate ligands, both highly expressed on the tumor cell surface, might be involved in tumor metastasis by mediating binding of blood-borne tumor cells via E- and/or P-selectin to vascular endothelium (8-11). In particular, E-selectin is postulated to be the most efficient selectin, mediating rolling of cancer cells to EC and SA-Le^a plays a major role as a ligand in the E-selectin dependent adhesion to EC. Cancer cells that express both SA-Le^a and SA-LeX undergo SA-Le^a mediated adhesion almost exclusively, possibly due to a higher affinity for the SA-Le^a structure or differential presentation of the oligosaccharide determinant. SA-Le^a specific MAbs including NS19-9, which we propose to use to identify SA-Le^a mimicking peptides, were inhibitory for adhesion of colon carcinoma cells to human umbilical cord vein endothelial cells. In addition, to interrupt the metastatic

process at the level of cellular adhesion, these compounds should interfere with blood vessel formation since E-selectin and SA-LeX are expressed on actively growing blood vessels.

E-selectin independent adhesion of tumor cells. Antibodies BR55-2 and BR15-6A directed to LeY significantly decreased the adhesion in a dose-dependent fashion, implicating the involvement of LeY in adhesion of these breast carcinoma cells. These data indicate that different human adenocarcinoma cells interact with EC via E-selectin-mediated and E-selectin-independent pathways in agreement with previously published reports that some invasive breast carcinoma cells do not interact with TNF-α-stimulated EC under dynamic conditions, but adhere to resting and stimulated EC via an E-selectin independent mode following static incubation (12,13).

Therapeutic inhibition of carbohydrate-protein interaction. Although all selectins share specificity for SA-LeX, the requirements for binding to E-selectin are distinct from those of P-selectin, because, for example PGSL-1 does not require tyrosine sulfation for binding to E-selectin (14). Unlike the case for P- and L-selectin, the presentation of SA-LeX and SA-Le^a ligands for E-selectin is not limited to mucin type glycoproteins since they are present on O-linked and N-linked oligosaccharides, as well as glycosphingolipids, suggesting mainly unmodified oligosaccharide recognition of E-selectin. Furthermore, SA-LeX was demonstrated to be able to sustain rolling of SA-Le^a-containing microspheres on E-selectin in a flow adhesion system (15). These observations support the key role of SA-LeX/SA-Le^a oligosaccharides as adhesion ligands for E-selectin. Thus, it is possible that in vivo blocking of selectin-dependent adhesion may even be more effective with ligand antagonists than with the native ligands and/or antagonists based on the protein of individual selectins, since it will affect binding of all selectin family members. Thus, instead of using receptors for screening of combinatorial libraries, we proposed to use MAbs that specifically recognize ligands for endothelial adhesion molecules. The advantage of peptide library screening with the MAb is that the peptides mimic respective carbohydrate that can display binding cross-reactivity for all selectins since all selectins display specificity for the same ligand. The crucial role of selectin-dependent neutrophil adhesion and carcinoma cell-adhesion in their recruitment process implies that in vivo blockage of selectin-dependent interaction can decrease leukocyte mobilization and the severity of the metastatic process, respectively. Fifty to 70% inhibition of neutrophil recruitment with monoclonal antibodies and selectin-Ig chimeras was demonstrated (16) comparable to L-selectin-deficient mice (17) in studies of a mouse model of peritonitis. Identification of the structure of selectin ligands has provided the opportunity to test oligosaccharide ligands and their derivatives for anti-inflammatory effect in vivo despite their rather low binding affinity to the receptor. Derivatives of SA-LeX oligosaccharide prevented P- and E-selectin-dependent inflammation in rodent models (24, 25) and in a cat model of myocardial perfusion injury (18). High-affinity ligands described to date for selectins are acidic polysaccharides such as single-stranded oligonucleotides (19), heparin (20), inositol polyanions (21), fucoidan (22) glycyrrhizin, a triterpene glycoside identified using a search of a three-dimensional database (23) as well as a peptide mimetic identified in random peptide library (24). However, this high-affinity peptide contains an identical sequence (QLWD) to the first four amino acids residues of PSGL-1, which binds to both P- and E-selectin, suggesting protein and not carbohydrate binding. Consequently, this peptide does not define a carbohydrate mimotope as described in this application.

Although these approaches have not been studied in tumor models in vivo as extensively as anti-inflammatories, administration of E-selectin-specific antibody and soluble E-selectin abrogated the formation of hepatic metastasis and lung colony formation in vivo (25-27). Further, E-selectin transgenic mice, in which E-selectin is expressed in all organs at high levels under the β-actin promoter developed metastasis in lung, liver and mesenteric lymph nodes, and died much faster than wild-type animals (28). These data imply that similar molecular mechanisms do indeed underlie inflammation and metastasis and that similar therapeutic approaches can be used to intervene with these processes.

METHODS AND PROCEDURES

As part of our effort to develop high-affinity novel molecules to inhibit the adhesion of adenocarcinoma cells to EC, we screened a 12-mer random peptide library for binding to MAbs specific for tumor-associated oligosaccharides SA-Le^a (NS19-9) (Wistar Institute), SA-LeX (FH6) (ATCC) and LeY (BR15-6A) (Wistar Institute). E-selectin-Ig chimeric protein was obtained from Dr. George Haevner

(Centocor Inc., Malvern, PA) was also used to identify peptide equivalents of cognate ligands that may involve other structural elements in addition to SA-LeX/SA-Le^a carbohydrates.

Random peptide library. In the library we have chosen, random peptides are displayed on the surface of bacteria within the structural context of the thioredoxin which is inserted into flagellin gene and the resulting fusion protein FLITR is expressed on the bacterial cell surface. This enables efficient isolation of these bacteria displaying peptides with affinity to immobilized antibodies or other binding proteins. An aliquot of FLITRX library containing at least 2 x 10^{10} cells to ensure full representation of peptides, was grown to saturation for 15 hr in IMC/amp100 medium (M9 medium with 1 mM $MgCl_2$, 0.5% glucose, 0.2% casamino acids and 100 μg/ml ampicilin). The culture of 10^{10} cells was diluted with fresh IMC/amp100 medium containing 100 μg/ml tryptophan and incubated for an additional 6 hr. The induced bacteria were panned on an MAb-coated (20 μg/ml) plate followed by blocking with 1% nonfat milk containing 150 mM NaCl and 1% α-methyl mannoside. After washing, cells were eluted and collected in 10 ml of fresh IMC/amp100 medium and are incubated at 25°C until saturation. The entire selection process was repeated four more times. Individual colonies were isolated on ampicilin-containing plates and grown in 2 ml rich media. Forty μl of culture was induced for protein expression in a 2 ml IMC/amp100 medium with tryptophan for 6 hr. The clones isolated with carbohydrate-specific MAb in the final selection cycle were tested for protein expression using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and identified after probing by Western blot with the respective MAb and with E-selectin-IgG (29). In addition, nitrocellulose filters after protein transfer were probed with MAb specific for thioredoxin to confirm the identity of bands detected with specific MAbs or E-selectin-Ig. Only clones which display strong signal identified on the Western blot with the respective MAb were selected for sequence identification. Expression of the FLITRX fusion protein containing peptide sequences mimicking carbohydrate structures by the bacterial clones was detected with the specific MAb or E-selectin-IgG fusion protein (10 μg/ml). The expression and the location of thioredoxin on the nitrocellulose filter is confirmed after incubation of parallel filters with trxA-specific MAb (anti-Thio™) (Invitrogen, Carlsbad, CA). Following antibody binding, the filters were washed, incubated with HRP-conjugated goat anti-mouse antibody and treated to a freshly mixed luminol and oxidizing solution for 1 min, air-dried and exposed on a Reflection™ film (NEN™, Life Sciences Products, Boston, MA).

DNA sequencing. DNA was isolated from the selected bacteria using standard isopropanol precipitation in the presence of potassium acetate using a mini-column DNA purification column (Qiagen, Chatsworth, CA). The phages selected from the third round of biopanning were precipitated with polyethylene glycol after amplification, and single-stranded DNA is prepared by phenol extraction. The nucleotide sequences of the DNA from the selected bacteria or phages were determined by the dideoxynucleotide chain termination method using specific primers at The DNA Facility at The Wistar Institute.

Titration of peptides with MAbs. The following experiments determined whether the isolated peptides retain conformational properties of carbohydrates in a series of in vitro assays: a) direct binding to respective MAbs and E-selectin-IgG in solid phase and inhibition of MAbs and E-selectin-IgG binding to the cognate carbohydrate ligands in solid phase.

Selected peptides were synthesized by standard solid-phase strategies and HPLC-purified at the Peptide Synthesis Facility of The Wistar Institute. The structures was confirmed by fast-atom bombardment mass spectrometry at The Wistar Institute Protein Sequencing Facility. The binding of MAbs to the immobilized peptide conjugates was measured with ELISA as described (30). Microtiter plates were coated in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ and 3 mM NaN_3 pH 9.6) by air-drying with aliquots of 1 nM to 1 mM peptide and allowed to bind overnight at 37°C. Dose-response studies for binding and inhibition of MAb binding to the cognate antigens were run at six concentrations, with sequential dilutions from the initial stock solution giving data points over more than a 5 log concentration range. To avoid non-specific binding, plates were incubated first with 10% γ-globulin-free horse serum in PBS for 1 hr. Peptides were then incubated with purified MAb (5 μg/ml in a blocking solution) for 1 hr, washed and incubated for 1 hr with goat anti-mouse Ig conjugated with HRP. The reaction was developed with 3,3'-diaminobenzidine in 50 mM Tris-HCl buffer, pH 7.6 containing 3% H_2O_2 for 10 min. The color developed was read at 450 nm after stopping the reaction with 1 M phosphoric acid. For peptide

competition assays, test peptides at concentrations ranging from 10 nM to 1 mM were preincubated with 100 µl of the respective MAbs (10 µg/ml) at room temperature. After 1 hr incubation, MAb/peptide mixtures were transferred to wells precoated with a constant amount of neoglycoprotein containing coupled multivalent carbohydrate determinants (SA-LeX or SA-Le^a PAA) (0.15 µg/well) and allowed to bind for 1 hr. Wells were washed and anti-mouse-HRP antibody and the enzyme substrate are added. IC_{50} was calculated by non-linear least-squares regression to a four-parameter logistic equation.

Peptide analogs with enhanced binding. Two approaches were applied to generate structural equivalents of carbohydrate ligands with enhanced affinity: 1) increase of the valency of peptides mimicking carbohydrate structures as discussed below and 2) random mutagenesis using peptide array.

Multivalent peptides . Multivalency is the critical feature for the interaction of carbohydrates on the cell surface with endolectins such as selectins. SA-LeX/SA-Le^a are presented as "oligosaccharide patches" on glycoproteins or on glycosphingolipids clustered on the cell surface, forming polyvalent determinants that enhance the affinity of the selectin-ligand interaction. Thus, particularly important for the development of reagents that will interfere with protein-carbohydrate interactions of EC in vivo is to design molecules with higher binding affinity than the authentic receptor that will be efficient in interrupting this interaction. The presentation of receptor antagonists in multivalent form such as multi-antigen peptide (MAP) should render them considerably more potent as adherence blockers. Multiple antigenic peptides containing eight copies of peptide #4 mimicking SA-Le^a on a branched lysine core was acquired commercially (Research Genetics, Huntsville, Inc., AL).

Peptide arrays. Individual substitutions of amino acids in positively identified dodecapeptides were made on a porous membrane (e.g. cellulose paper) by SPOT-Synthesis (31). This method provides the positionally addressable, parallel chemical synthesis. Briefly, a droplet of liquid applied onto a cellulose membrane is absorbed and spreads as a spot which can form a reactor for chemical conversions. This membrane is stable for 10-20 repeated assays and can be used for Western Blotting. The peptide array are generated in a laboratory of our collaborator in the project, Dr. L. Otvos at the Wistar Institute. We have already obtained a peptide array consisting of 204 dodecamer based on the sequence of peptide #4 mimicking SA-Le^a identified by panning with MAb 19-9 which was analyzed by probing membrane with MAbs. The membrane is blocked with 5% milk in PBS-T (pH 6.8) and is allowed to bind the primary antibody in 10 µg/ml for 1 hr. The membrane is washed and treated with HRP-conjugated IgG. Then the binding is detected with the chemiluminescence reagent (NEN) exposure onto a film and the subsequent development. We are currently examining the binding affinity on the peptide array generated from the dodecapeptide selected with E-selectin IgG fusion protein.

Characterization of cell surface expression of SA-LeX, SA-Le^a and LeY. To evaluate the ability of the peptides to interfere with the function of endothelial adhesion molecules will be evaluated in syngeneic in vivo model of metastasis. We have characterized the expression of carbohydrate structures in a panel of murine tumor cells.

FACS. Cells are maintained in DMEM containing 10% FBS and 2 mM glutamine. Expression of carbohydrate antigens was determined with specific MAbs using flow cytometry at The Wistar Institute Flow Cytometry Facility. A single-cell suspension was prepared from adherent cultures by brief trypsinization. Two x 10^5 cells were stained with a 10 µg/ml solution of MAb in PBS followed by goat anti-mouse fluorescein-conjugated antibody. Incubations with antibodies were for 30 min at room temperature. After the final wash, cells were resuspended in buffer containing 1 µg/ml of propidium iodide to gate out dead cells selectively. Isotype control antibodies were used to determine background staining.

Transfection. Murine tumor cell line expressing SA-Le^a determinant was generated by transfection of B16F10 cells with α1,3/4-fucosyltransferase (32). Murine B16 melanoma cells were transfected with vector containing the hygromycin resistance gene only or with vector πH3M containing α1,3/4-FTIII. Transfectants were generated by electroporation (Gene Pulser, Bio-Rad Laboratories, Richmond, CA) at 250 V and 960 mF in 4-mm electroporation cuvettes. Clones were selected for hygromycin resistance in 10% FCS/IMDM containing 500 µg/ml hygromycin (Boehringer Mannheim Corp.) and tested for antigen expression by flow cytometry. Using the same transfection procedure MethA

cells will be transfected with pCDNA expression vector containing α1,2-FT cDNA to generate murine cell line expressing LeY.

Experimental inflammatory model. The accumulation of neutrophils is a characteristic feature of acute and chronic inflammatory disease and early steps in the recruitment of these cells to the site of inflammation, depends upon E-selectin-mediated interaction. Thus, inhibition of neutrophil recruitment in vivo is an important test of the ability of potential therapeutic agents to inhibit E-selectin-mediated events. To determine the ability of isolated peptides to inhibit E-selectin function in vivo, the acute inflammatory condition was induced with zymosan injected intraperitoneally (i.p.) into mice followed 3 hr later by an intravenous (i.v.) injection of peptide #4 (1 mg) (33). Neutrophils were harvested and counted 1 hr later. In control experiments the same dose of Lewis Y mimicking peptide, which does not bind to NS-19-9 MAb was used.

RESULTS

Peptides mimicking carbohydrate structures. Toward the development of novel molecules to inhibit the adhesion of human breast adenocarcinoma cells to EC and ultimately metastasis in vivo, we have characterized peptides derived from a 12-mer random peptide library for binding to antibodies developed in our laboratory, specific to tumor-associated oligosaccharides, LeY (MAb BR15-6A) (34), SA-Le^a (MAb NS19-9) (4) and SA-LeX (MAb FH6) (35), as well as E-selectin-immunoglobulin chimeric protein (36).

We have sequenced DNA encoding peptide of several bacterial clones that bind to the SA-Le^a structures using MAbs NS19-9 and E-selectin-IgG and Lewis Y using MAb BR15-6A. The highly conserved core region of each consensus sequence usually is 3 to 5 amino acid long and positional preference of these consensus residues within the dodecapeptide varies. Such positional preference might be a reflection of structural constraints of the inserted peptide imposed by thioredoxin which might limit the position for the antibody binding. Further examination of the sequences reveals that the peptides isolated by BR15-6A and NS19-9 MAbs and E-selectin-IgG fell into distinct consensus-sequence groups that discriminated between the MAbs and the lectin, and that the native carbohydrate antigens LeY and SA-Le^a and the peptides bound only to the MAb or lectin protein used for their isolation.

SA-Lewis a. Two distinct consensus sequences GXWXXVLEG and VVGXP were identified in families of peptides isolated with MAb NS19-9, which recognizes E-selectin ligand, SA-Le^a. The structural relevance of these motifs is unclear. These motifs may indicate the following: i) peptides based on two different motifs isolated with the same MAb can mimic different structural topographies of the cognate SA-Le^a carbohydrate; ii) these subset of peptides may very likely represents nonoverlapping surfaces of cognate antigen, and iii) peptide binding to MAb occurs at separate sites. Peptide #4 from NS19-9 family II mimicking SA-Le^a ligand was selected for further studies based on the presence of consensus motif which appeared in multiple bacterial isolates after five rounds of panning (Table 1).

Table 1. Peptide sequence families mimicking SA-Le^a carbohydrate structure.

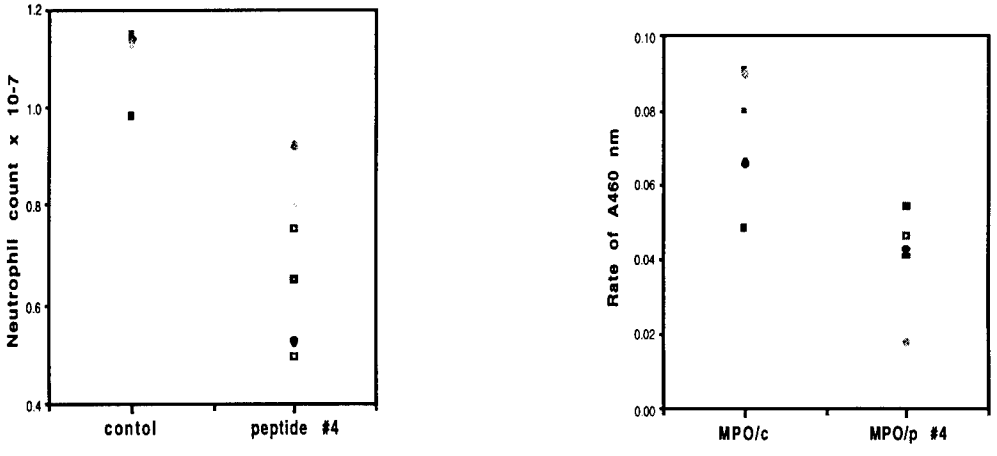
I	#2:	VG IWSVVSEGSR	II	#1 RCSVGVPFTMES
	#3:	QDGVWEHVLEGG		#4: DLWDWVVGKPAG
	#15	VLSGRGGLCTW		#12: VIGAASHDEDVD
	#18:	TIEPVLAEMFMG		#14: DKETFELGLFDR
				#15: FSGVRGVYESRT
				#19: PDDAPMHSTRVE

Peptide #4 demonstrated dose-dependent binding with MAb in enzyme-linked immunosorbent assay (ELISA) (data not shown). This peptide was also inhibitory for MAb NS19-9 binding to the synthetic SA-Le^a-PAA neoglycoprotein (Glycotech, Inc., Rockville, MD) which contains the cognate oligosaccharide determinant, implying that the sequence DLWDWVVGKPAG represents a solvent-accessible epitope. The 50% inhibitory concentration (IC_{50}) value of peptide #4 was calculated at 700 μM. Control peptide mimicking Lewis Y carbohydrate structure failed to block antibody binding, indicating that

the inhibitory effects of the native sequences are not due to nonspecific effects. Although, the peptide #4 shows relatively low-affinity binding with the antibody, a strong binding signal was observed on the Western blots when the peptide sequence is expressed in the context of thioredoxin. This might be a reflection of structural constraints of the inserted peptide imposed by thioredoxin as compared with the conformation of the peptide in solution or in solid phase.

Acute inflammation model in vivo. An interaction with SA-Le^a, is not relevant for adhesion of neutrophils, since it is not expressed on their surface. In tumor cells, however, E-selectin-mediated interaction with SA-Le^a expressed on human cancer cells is preferentially used over SA-LeX to adhere to EC. Nevertheless, because SA-Le^a binds to E-selectin, we tested whether administration of a SA-Le^a mimicking molecule would diminish the influx of neutrophils into chemically irritated peritoneum in vivo. Peptide treatment significantly ($P<0.001$) reduced the number of neutrophils in peritoneal lavage fluids (Fig. 1A). Control experiments using the same dose of Lewis Y mimicking peptide, which does not bind to NS-19-9 MAb, showed no decrease in neutrophil influx relative to PBS-injected mice.

To confirm these results, myeloperoxidase (MPO) activity, which is an enzymatic marker for neutrophils, was measured spectrophotometrically as an absorbance rate in the homogenates of collected cells (33). Significant reduction of enzymatic activity ($P<0.005$) was observed in parallel with decreased neutrophil numbers assessed by total neutrophil count (Fig. 1B). These results strongly suggest that the reduction in enzyme activity is due to reduction in neutrophil recruitment in mice treated with peptide mimicking E-selectin ligand SA-Le^a and that this peptide can inhibit E-selectin function in vivo.



A B

Fig. 1. Reduction of neutrophil influx upon administration of peptide #4 mimicking SA-Le^a carbohydrate in mice with chemically induced peritonitis. Results are from 4 experiments (3-4 mice in each group). A, Neutrophil counts; B, MPO activity in collected neutrophils. Unrelated peptide was administered in control mice. Statistical analysis using nonparametric unpaired t-test gave P values <0.001 and <0.005 for data in A and B, respectively.

Characterization of essential amino-acids using peptide array. An array of 204 peptides was prepared based on the amino acid (aa) sequence of peptide #4 isolated from the family 1 with MAb NS19-9 with all point aa substitutions within the sequence DLWDWVVGKPAG and measured binding of MAb (Fig. 2). An analysis of the peptide array suggest that most amino acid (aa) substitutions within C-terminal were tolerated and did not influenced MAb binding, whereas critical residues were clearly identified within the N-terminal. Multiple substitutions of LWD and W at the positions 2 to 5 abolished MAb binding with W at position 3 being the most critical, since the least of aa substitutions at this position were tolerated by the MAb NS19-9. Substitutions with H, Y, A, D and S completely abolished the binding whereas M significantly decreased the MAb binding. Similarly, substitutions of second W at the position 5 with H, A, R and K completely abolished the binding. Most of the substitutions upstream from the

position 5 were allowed for MAb binding and no preference for aa was evident from the substituted peptides within the C-terminal. Furthermore, crossreactive peptides, which were formed by multiple aa substitutions were identified. Peptides substituted with F at the 5th position and E at 8th and 9th positions showed significantly stronger signal on the peptide array (Fig. 2), suggesting that we have identified aa sequences that showed higher binding affinity as compared to the original peptide and that can act as better structural mimics than the peptides identified using combinatorial peptide library.

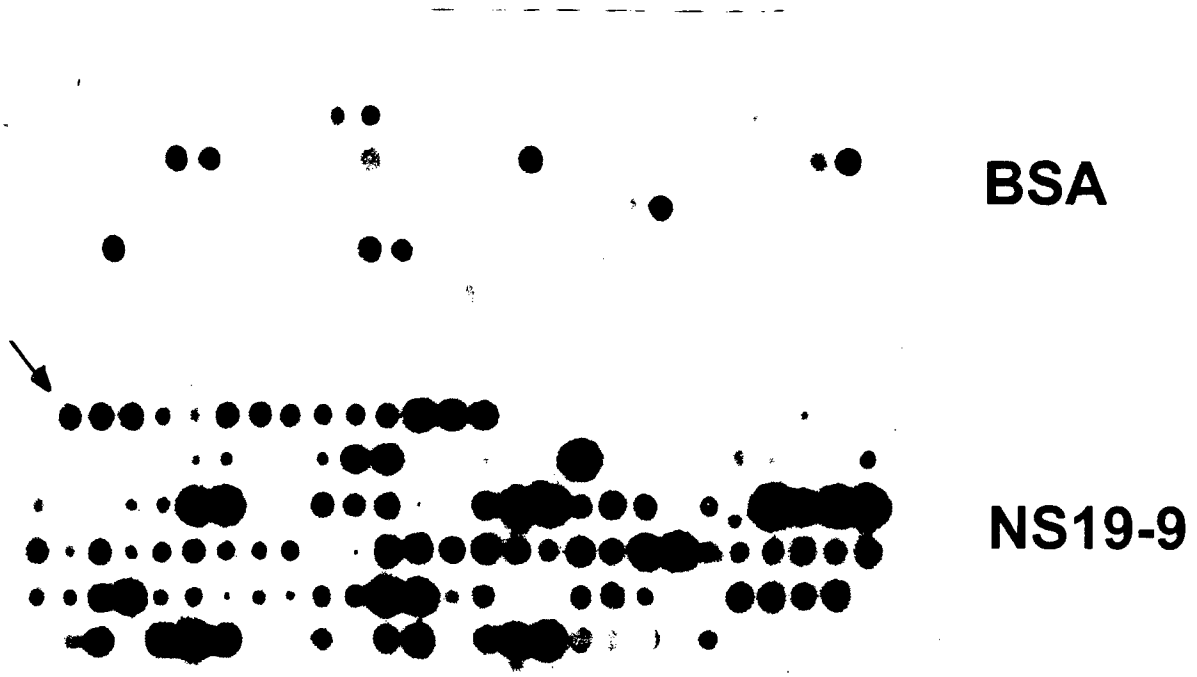


Fig. 2. Peptide array generated by substitutions of all individual aa of peptide #4 mimicking SA-Le^a selected from combinatorial peptide library using MAb NS19-9 and treated for the antibody binding. Arrow points spot in the array that represents binding to the original peptide #4. Control identical array was blotted with BSA instead MAb NS19-9 followed by HRP-conjugated anti mouse antibody.

E-selectin ligand(s). To identify peptides mimicking E-selectin ligand, that may involve other than carbohydrate epitopes of the natural ligand, we have initiated screening of the library directly with E-selectin-IgG fusion protein. Among isolated peptides the motif VLSP was found in 7 of 16 clones recovered. In addition, preference for V/G and RR in the sites flanking the 5- amino acid motif were also found. In another group of peptides, a PGR sequence was found in 3 clones. As discussed above, the peptides with different sequence motifs may mimic different topographical surfaces of the cognate carbohydrates or bind to different binding areas of E-selectin combining site.

Lewis Y. Peptide from the family isolated with MAb BR15-6A that inhibits adhesion of human tumor cells to EC in E-selectin independent mode demonstrating sequence motif RPDL, mimicking the LeY carbohydrate was identified using combinatorial library approach. Peptide #7 from the family of LeY-mimicking peptides was selected based on a consensus motif which appeared in multiple bacterial isolates after five rounds of panning with the BR15-6A MAb. In vitro studies demonstrated using ELISA that the identified sequence DEVRPDLISTEE represents a putative epitope for the MAb but was not recognized by E-selectin-IgG (data not shown).

In summary, the results suggest that the conformation of the peptides can mimic the topography of the epitopes recognized by MAbs and E-selectin. Data implicate further that the carbohydrate mimotopes are effective in vivo blockade of selectin-dependent interaction, which can diminish leukocyte recruitment in inflammatory process. The data point to the feasibility of using small molecule antagonists isolated from

the combinatorial peptide library to block E-selectin function in vivo and will also prove useful to inhibit metastatic process.

In vivo tumor models.

E-selectin-dependent adhesion. An syngeneic model of tumor metastasis was proposed for in vivo studies, in which murine tumor cells derived from the same murine background will be inoculated to form metastasis. We have characterized a panel of human and murine adenocarcinoma cell lines with respect to the expression of the tumor-associated antigens SA-LeX and SA-Le^a. MethA fibrosarcoma (kindly provided by Dr. A. DeLeo, University of Pittsburgh), Gl261 glioblastoma, CT-26 colon carcinoma, B16F1 and B16F10 melanoma clones (DCTDC Tumor Repository, NCI, Frederick, MD) and breast adenocarcinoma 66.1, JC, 410.1 cell lines (provided by Dr. A. Fulton, University of Maryland, Baltimore, MD). All cell lines express SA-LeX except B16F1 and B16F10 variants, whereas no SA-Le^a expression was observed among all cell lines. To extend the syngeneic model to tumors expressing SA-Le^a structure, we generated murine tumor cell line stably expressing specific glycosyltransferase, which activity results in synthesis of carbohydrate determinants SA-Le^a α1,3/4 fucosyltransferase can mediate synthesis of both ligands, since it is specific for type 1 and 2 lactotetraosyl chain structures, which constitute the core chain of Lewis structures. Thus, the presence of the acceptor for this glycosyltransferase within the cells should guarantee that transfection results in biosynthesis of one or both structures. We have established that B16F10 cell line synthesize type 1 lactoseries acceptor structures. The expression vectors containing cDNA of α1,3/4 fucosyltransferase (α1,3/4FTIII) (provided by Dr. Brian Seed, Massachusetts General Hospital, Boston, MA).

E-selectin-independent adhesion of adenocarcinoma cells. To generate murine tumor cell line expressing LeY we are currently transfecting MethA and Gl261 cell lines with α1,2-fucosyltransferase (α1,2FT) available from our previous studies. If LeY expressing murine tumor cells can not be generated, we have characterized a panel of human carcinoma cells expressing LeY, which will be used to establish tumor model in immunodeficient mice. We have demonstrated that human breast adenocarcinoma cell lines, SKBR5 and SKBR3 but not colon adenocarcinoma cells LS180, SW1116 and SW948, adhere to TNF-α-stimulated EC and this process is mediated by E-selectin as determined by the inhibitory effect of anti-E-selectin MAb as well as NS19-9 MAb specific for SA-Le^a (not shown).

CONCLUSIONS

SA-LeX and SA-Le^a carbohydrate structures are expressed on leukocytes and most human carcinoma cells. Several studies have implicated E-selectin and carbohydrate ligands in leukocytes and carcinoma cell adhesion to the endothelium, an interactions thought to be required for inflammation and tumor extravasation during metastasis. The overall objective of this project was to identify the peptides that mimic oligosaccharide receptors for E-selectin, i.e. SA-LeX and SA-Le^a and LeY, which presumably mediated E-selectin independent adhesion of tumor cells to EC and elucidate their effect on tumor cells adhesion to endothelial cells in vitro and abrogate tumor metastasis in vivo.

For the identification of high-affinity inhibitors for E-selectin-mediated tumor cell adhesion we proposed to screen using specific MAbs of recombinant peptide library display technology that has a demonstrated utility in discovering ligands for important therapeutic targets. Highly diverse peptide libraries offer many distinct advantages over difficult chemical or enzymatic synthesis of complex carbohydrates, providing a notably inexpensive and rapid identification and optimization of novel ligands. The binding affinities of the originally identified peptides are K_D values at mid-micromolar range, which is comparable with the values obtained for soluble oligosaccharide SA-LeX binding to bivalent E-selectin and with carbohydrate lectin interactions. The IC_{50} for SA-LeX binding to E-selectin is 750 nM in competition with bivalent E-selectin-Ig fusion protein binding to immobilized glycoconjugates containing SA-LeX, and a K_D of ~1.2 mM has been measured by transferred nuclear Overhauser enhancement measurements. Introducing a novel technology such as peptide array into the studies we demonstrated that it provides not only fast method to identify aa residues critical for binding but also to modify the peptides in order to improve their binding ability and efficacy to block protein-ligand interaction. Peptide array was demonstrated to represent powerful method to identify aa substitutions to develop better mimics of

carbohydrate structures. In addition to the originally proposed in a grant application MAbs we applied for screening of the peptide library E-selectin-IgG chimeric protein to identify mimics of other than SA-Le^a and SA-LeX epitopes or ligands. Our results thus far demonstrate that it is possible to isolate carbohydrate mimics that are effective in vivo blockade of selectin-dependent interaction, which can diminish leukocyte recruitment in inflammatory process. The selected peptides mimicking E-selectin ligands are currently tested to confirm their ability to block metastatic process in vivo using murine syngeneic tumors expressing E-selectin ligands that we have established.

In summary, we demonstrated that the use of combinatorial peptide analog technology in combination with synthetic peptide array allow us to identify improved antagonists of E-selectin and other adhesion molecules, which may lead to the further development of agents with greatly enhanced therapeutic potential.

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LIST OF PERSONNEL RECEIVING PAY FROM THIS EFFORT

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